COMPOSITIONS AND METHODS FOR THE TREATMENT OF SEPSIS

FIELD OF THE INVENTION

The present invention relates to compositions and methods for the prevention and treatment of blood-borne and toxin-mediated diseases, and in particular anti-C5a antibodies for the prevention and treatment of sepsis in humans as well as other animals.

BACKGROUND OF THE INVENTION

Sepsis is a major cause of morbidity and mortality in humans and other animals. It is estimated that 400,000-500,000 episodes of sepsis resulted in 100,000-175,000 human deaths in the U.S. alone in 1991. Sepsis has become the leading cause of death in intensive care units among patients with non-traumatic illnesses. [G.W. Machiedo et al., Surg. Gyn. & Obstet. 152:757-759 (1981).] It is also the leading cause of death in young livestock, affecting 7.5-29% of neonatal calves [D.D. Morris et al., Am. J. Vet. Res. 47:2554-2565 (1986)], and is a common medical problem in neonatal foals. [A.M. Hoffman et al., J. Vet. Int. Med. 6:89-95 (1992).] Despite the major advances of the past several decades in the treatment of serious infections, the incidence and mortality due to sepsis continues to rise. [S.M. Wolff, New Eng. J. Med. 324:486-488 (1991).]

Sepsis is a systemic reaction characterized by arterial hypotension, metabolic acidosis, decreased systemic vascular resistance, tachypnea and organ dysfunction. Sepsis can result from septicemia (i.e., organisms, their metabolic end-products or toxins in the blood stream), including bacteremia (i.e., bacteria in the blood), as well as toxemia (i.e., toxins in the blood), including endotoxemia (i.e., endotoxin in the blood). The term "bacteremia" includes occult bacteremia observed in young febrile

children with no apparent foci of infection. The term "sepsis" also encompasses fungemia (*i.e.*, fungi in the blood), viremia (*i.e.*, viruses or virus particles in the blood), and parasitemia (*i.e.*, helminthic or protozoan parasites in the blood). Thus, septicemia and septic shock (acute circulatory failure resulting from septicemia often associated with multiple organ failure and a high mortality rate) may be caused by a number of organisms.

The systemic invasion of microorganisms presents two distinct problems. First, the growth of the microorganisms can directly damage tissues, organs, and vascular function. Second, toxic components of the microorganisms can lead to rapid systemic inflammatory responses that can quickly damage vital organs and lead to circulatory collapse (*i.e.*, septic shock) and oftentimes, death.

There are three major types of sepsis characterized by the type of infecting organism. Gram-negative sepsis is the most common and has a case fatality rate of about 35%. The majority of these infections are caused by *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Gram-positive pathogens such as the *Staphylococci* and *Streptococci* are the second major cause of sepsis. The third major group includes fungi, with fungal infections causing a relatively small percentage of sepsis cases, but with a high mortality rate.

Many of these infections are acquired in a hospital setting and can result from certain types of surgery (*e.g.*, abdominal procedures), immune suppression due to cancer or transplantation therapy, immune deficiency diseases, and exposure through intravenous catheters. Sepsis is also commonly caused by trauma, difficult newborn deliveries, and intestinal torsion (especially in dogs and horses).

Many patients with septicemia or suspected septicemia exhibit a rapid decline over a 24-48 hour period. Thus, rapid methods of diagnosis and treatment delivery are essential for effective patient care. Unfortunately, a confirmed diagnosis as to the type of infection traditionally requires microbiological analysis involving inoculation of blood cultures, incubation for 18-24 hours, plating the causative organism on solid media, another incubation period, and final identification 1-2 days later. Therefore,

therapy must be initiated without any knowledge of the type and species of the pathogen, and with no means of knowing the extent of the infection.

It is widely believed that anti-endotoxin antibody treatment administered after sepsis is established may yield little benefit because these antibodies cannot reverse the inflammatory cascade initiated by endotoxin. In addition, the high cost of each antibody could limit physicians' use of a product where no clear benefit has been demonstrated. [K.A. Schulman et al., JAMA 266:3466-3471 (1991).] Furthermore, these endotoxin antibodies only target gram-negative sepsis, and no equivalent antibodies exist for the array of gram-positive organisms and fungi.

Clearly, there is a great need for agents capable of preventing and treating sepsis. It would be desirable if such agents could be administered in a cost-effective fashion. Furthermore, approaches are needed to combat all forms of sepsis.

SUMMARY OF THE INVENTION

The present invention relates to compositions and methods for the prevention and treatment of blood-borne and toxin mediated diseases, and in particular anti-C5a antibodies for the prevention and treatment of sepsis in humans as well as other animals.

The present invention provides a composition comprising antibody specific for complement component C5a peptide. In another embodiment, the composition comprises antibody which is specific for complement component C5a peptide, wherein the C5a peptide has a C-terminal region and an N-terminal region, and the antibody is not reactive with the C-terminal region. In further embodiments, the antibody is specific for the N-terminal region of complement component C5a peptide. In an additional embodiment, the antibody is also not reactive with complement component C5 protein.

It is not intended that the present invention be limited to antibodies specific for C5a peptides from certain animals. In certain embodiments, the antibody is specific for rat C5a peptide. In other embodiments, the antibody is specific for bovine C5a

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peptide. In still other embodiments, the antibody is specific for porcine C5a peptide. In a preferred embodiment, the antibody is specific for human C5a peptide.

It is also not intended that the present invention be limited to antibodies generated in a particular animal. A variety of animals are useful for generating the antibodies of the present invention. In one embodiment, the antibody is generated in an animal selected from a mouse, a rat, a horse, a goat, a chicken, and a rabbit. In some embodiments, the antibodies are collected from the blood of the animal. In other embodiments, the animal generating the antibodies is a bird, and the antibodies are collected from egg yolk.

It is not intended that the present invention be limited to the nature of the antibodies, as a variety of antibody types are contemplated. In one embodiment, the antibodies are monoclonal. In another embodiment, the antibodies are humanized. In other embodiments, the antibodies are chimaeric. In a preferred embodiment, the antibodies are polyclonal.

The present invention also provides a method of producing polyclonal antibody. In one embodiment, the method comprises, providing; an animal and an immunogenic composition, wherein the composition comprises C-terminal truncated C5a peptides; and immunizing the animal with the immunogenic composition in order to generate antibodies. In some embodiments, the immunogenic composition comprises adjuvant. In a further embodiment, antibodies are collected from the animal.

It is not intended that the present invention be limited to antibodies specific for C5a peptides from any particular animal. In certain embodiments, the antibody is specific for rat C5a peptide. In other embodiments, the antibody is specific for bovine C5a peptide. In still other embodiments, the antibody is specific for porcine C5a peptide. In a preferred embodiment, the antibody is specific for human C5a peptide.

It is not intended that the present invention be limited to particular C-terminal truncated peptides. A variety of C-terminal truncated peptides are contemplated. In one embodiment, the C-terminal truncated peptide corresponds to the entire N-terminal region of C5a peptide. In another embodiment, the C-terminal truncated peptide corresponds to the entire N-terminal region of C5a peptide and a portion of the C-

terminal region. In another embodiment, the C-terminal truncated peptide is a fragment or portion of the N-terminal region of C5a peptide. In another embodiment, the C-terminal truncated C5a peptide is between approximately 5 and 50 amino acids in length. In some embodiments, the C-terminal truncated peptide is approximately fifty amino acids in length. In other embodiments, the C-terminal truncated peptide is approximately five amino acids in length. In preferred embodiments, the C-terminal truncated peptides are 20 amino acids in length. In certain embodiments, the C-terminal truncated peptides are selected from SEQ ID NOS:2, 4, 5, 14, 15, and 16.

The present invention also provides a method of treating a subject with the antibodies of the present invention. In one embodiment, the method comprises; providing; a subject, and a therapeutic composition comprising an antibody specific for complement component C5a peptide, wherein the C5a peptide has a C-terminal region and an N-terminal region, and wherein the antibody is not reactive with the C-terminal region; and administering the therapeutic composition to the subject. In another embodiment, the antibody is specific for the N-terminal region of complement component C5a peptide.

In one embodiment, the present invention provides a method comprising; providing; a subject, and a therapeutic composition comprising an antibody specific for complement component C5a peptide, wherein the C5a peptide has a C-terminal region and an N-terminal region, and wherein the antibody is not reactive with the C-terminal region; and administering the therapeutic composition to the subject. In another embodiment, administering the therapeutic composition reduces the binding of complement component C5a peptide to one or more neutrophils of the subject. In a certain embodiment, administering the therapeutic composition reduces bacteremia in the subject. In yet another embodiment, administering the therapeutic composition increases the H_2O_2 production of neutrophils of the subject. In a preferred embodiment, administering the therapeutic composition reduces the symptoms of sepsis.

It is not intended that the therapeutic method of the present invention be limited to particular subjects. A variety of subjects are contemplated. In one

embodiment the subject is selected from a pig, a rat, a cow, a horse, and a human. In one embodiment, the therapeutic composition is administered to a subject suffering from symptoms of sepsis. In another embodiment, the therapeutic composition is administered prophylactically to a subject at risk for sepsis, including new born humans and animals.

It is not intended that the therapeutic method of the present invention be limited to certain modes of administration. A variety of modes of administering the therapeutic composition are contemplated. In one embodiment, the therapeutic composition is administered by a mode selected from intravenously, intra-muscularly, subcutaneously, intradermally, intraperitoneally, intrapleurally, intrathecally, and topically.

It is not intended that the present invention be limited to a particular therapeutic composition. A variety of compositions are contemplated. In one embodiment the therapeutic composition comprises a soluble mixture of anti-C5a antibodies. In another embodiment, the anti-C5a antibodies are provided together with physiologically tolerable liquid, gels, solid carriers, diluents, adjuvants or excipients, and combinations thereof. In other embodiments, the therapeutic composition comprises anti-C5a antibodies and other therapeutic agents (e.g. other immunoglobulins or antibiotics).

The present invention also provides a method for screening C-terminal truncated C5a peptides to identify immunogens for the production of anti-C5a antibodies. In one embodiment, the method comprises, providing a C-terminal truncated C5a peptide, modifying the amino acid sequence of said C-terminal truncated C5a peptide, and screening said C-terminal truncated C5a peptide to identify immunogens for the production of anti-C5a antibodies. In one embodiment, the C-terminal truncated C5a peptide which is provided is selected from SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:14, SEQ ID NO: 15, and SEQ ID NO:16. In other embodiments, the screening step involves a chemotaxis assay (See e.g. Examples 7, 8 and 11). In a different embodiment, the screening step involves a competitive binding assay (See e.g. Examples 10 and 11). In an additional embodiment, the

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screening step involves administering the C-terminal truncated peptides to septic animals (*See e.g.* Example 11).

In another embodiment, the present invention provides a method for sepsis rescue, comprising: a) providing; i) a patient presenting symptoms of sepsis, ii) a therapeutic composition comprising an antibody specific for the complement component of a C5a peptide region, wherein said C5a peptide region is some fraction of the complete C5a peptide; and b) administering said therapeutic composition to said patient.

In a preferred embodiment, the C5a peptide region (recited in the sepsis rescue method above) is selected from the group consisting of the peptides defined by SEQ ID NO:2 and SEQ ID NO:75.

In another embodiment, the patient (recited in the sepsis rescue method above) presents the symptoms of sepsis for a period in the range of approximately six to twelve hours prior to the administration of said therapeutic composition.

In another embodiment, the patient (recited in the sepsis rescue method above) is selected from the group consisting of human, rat, cow, and pig.

In another embodiment, the antibody (recited in the sepsis rescue method above) is polyclonal or monoclonal and are not reactive with complement component C5.

DESCRIPTION OF THE FIGURES

Figure 1 shows survival curves of septic rats with and without the administration of anti-C5a antibodies.

Figure 2 shows a graph demonstrating the ability of anti-C5a antibodies to reduce bacteria blood of septic rats.

Figure 3 shows a graph demonstrating the ability of anti-C5a antibodies to reduce bacteria in the organs of septic rats.

Figure 4 shows a graph demonstrating the ability of anti-C5a antibodies to increase the level of H_2O_2 production in neutrophils of septic rats.

Figure 5 shows a graph demonstrating the ability of synthetic peptides to reduce human C5a-induced chemotaxis of neutrophils.

Figure 6 shows the chemotactic activity of KLH-linked synthetic peptides of human C5a peptide.

5   Figure 7 shows polyclonal rabbit anti-human C5a reactivity with regions of human C5a peptide.

Figure 8 shows the amino acid sequence of human C5a peptide and various smaller portions of the human C5a peptide.

Figure 9 shows a graph demonstrating the ability of certain synthetic peptides

10   to inhibit the binding of human C5a peptide to human neutrophils.

Figure 10 presents data demonstrating that PMA elicits a strong H_2O_2 response, which is inhibitable by the C5a peptide, and to varying degrees by peptides A, M, and C.

Figure 11 projects the image of a Western Blot presents data showing that the

15   polyclonal anti-human C5a antibody was able to recognize both human and rat C5a peptide.

Figure 12A-C present graphs demonstrating sepsis rescue via the administration of C5a antibody to reverse sepsis symptoms (as compared to control) at various timepoints following CLP (Cecal Ligation Puncture).

20   Figure 13 projects the complete amino acid sequence of Human C5a.

Figure 14 projects the complete amino acid sequence of Rat C5a.

Figure 15A projects the "M" fraction of Human C5a as defined by amino acids 21-40 (vis-a-vis the complete amino acid sequence of Human C5a).

Figure 15B presents a variant to the "M" fraction of Human C5a (as compared

25   to Figure 15A) wherein the serine at amino acid 27 is substituted for a cysteine.

Figure 16 projects the "C" fraction of Human C5a as defined by amino acids 55-74 (vis-a-vis the complete amino acid sequence of Human C5a).

Figure 17 projects the "M" fraction of Rat C5a as defined by amino acids 17-36 (vis-a-vis the complete amino acid sequence of Rat C5a).

Figure 18 projects the "C" fraction of Rat C5a as defined by amino acids 58-77 (vis-a-vis the complete amino acid sequence of Rat C5a).

Figure 19 projects the nucleic acid sequence of a Human C5a analog set out in GenBank (NCBI gibbsq 170109).

DEFINITIONS

As used herein, the term "patient" includes members of the animal kingdom including but not limited to human beings.

The phrase "symptoms of sepsis" refers to any symptoms characteristic of a subject with sepsis including but not limited to, increased respiration, increased heart rate, reduced arterial CO_2 saturation, arterial hypotension, metabolic acidosis, fever, decreased systemic vascular resistance, tachypnea and organ dysfunction (as manefest by, but not limited to, elevated transaminase, creatinine, and blood urea nitrogen). Sepsis can result from septicemia (*i.e.*, organisms, their metabolic end-products or toxins in the blood stream), including bacteremia (*i.e.*, bacteria in the blood), as well as toxemia (*i.e.*, toxins in the blood), including endotoxemia (*i.e.*, endotoxin in the blood). The term "sepsis" also encompasses fungemia (*i.e.*, fungi in the blood), viremia (*i.e.*, viruses or virus particles in the blood), and parasitemia (*i.e.*, helminthic or protozoan parasites in the blood). Thus, phenotypes associated with septicemia and septic shock (acute circulatory failure resulting from septicemia often associated with multiple organ failure and a high mortality rate) are symptoms of sepsis.

As used herein, the phrase "sepsis rescue" refers to the abatement of any one of the symptoms of sepsis (as defined above) in a patient presenting any one of the symptoms of sepsis wherein said septic patient is subsequently administered a therapeutic composition comprising an antibody specific for the complement component of a C5a peptide region, wherein said C5a peptide region is some fraction of the complete C5a peptide

The phrase "reduces the symptoms of sepsis" refers to a qualitative or quantitative reduction in detectable symptoms, including but not limited to a detectable impact on the rate of recovery from disease.

The phrase "at risk for sepsis" in reference to a subject is herein defined as a subject predisposed to the development of sepsis by virtue of the subject's medical status, including but not limited to such factors as infection, trauma (*e.g.,* abdominal perforation, such as by a gun shot wound), surgery (*e.g.,* intestinal surgery), and invasive procedures (*e.g.,* placement of a catheter, *etc.*) and the like.

As used herein, the term "antigen" refers to any agent (*e.g.,* any substance, compound, molecule [including macromolecules], or other moiety), that is recognized by an antibody, while the term "immunogen" refers to any agent (*e.g.,* any substance, compound, molecule [including macromolecules], or other moiety) that can elicit an immunological response in an individual. These terms may be used to refer to an individual macromolecule or to a homogeneous or heterogeneous population of antigenic macromolecules. It is intended that the term encompasses protein and peptide molecules or at least one portion of a protein or peptide molecule, which contains one or more epitopes. In many cases, antigens are also immunogens, thus the term "antigen" is often used interchangeably with the term "immunogen." The substance may then be used as an antigen in an assay to detect the presence of appropriate antibodies in the serum of the immunized animal.

The term "specific for" when used in reference to the interaction of an antibody and a protein or peptide means that the interaction is dependent upon the presence of a particular structure (*i.e.,* the antigenic determinant or epitope) on the protein; in other words the antibody is recognizing and binding to a specific protein structure rather than to proteins in general (*i.e.* non-specific or background binding).

The term "not reactive with" when used in reference to the potential interaction of an antibody and a protein or peptide means that the antibody does not recognize or bind specifically to that particular protein (*i.e.* binding is at background levels).

The term "operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function. Thus, control sequences operably linked to a coding sequence are capable of effecting the expression of the coding sequence. The control sequences need not be contiguous with the coding sequence, so long as they function to direct the expression

As used herein, the phrase "anti-C5a antibody" refers to antibodies which are specific for complement component C5a peptide, or portions thereof.

As used herein, the term "adjuvant" is defined as a substance known to increase the immune response to other antigens when administered with other antigens. If adjuvant is used, it is not intended that the present invention be limited to any particular type of adjuvant -- or that the same adjuvant, once used, be used all the time. It is contemplated that adjuvants may be used either separately or in combination. The present invention contemplates all types of adjuvant, including but not limited to agar beads, aluminum hydroxide or phosphate (alum), Incomplete Freund's Adjuvant, as well as Quil A adjuvant commercially available from Accurate Chemical and Scientific Corporation, Gerbu adjuvant also commercially available (GmDP; C.C. Biotech Corp.), and bacterin (*i.e.,* killed preparations of bacterial cells).

As used herein, the term "N-terminal region of C5a peptide" refers to the N-terminal 50% of the complement component C5a peptide.

As used herein, the term "C-terminal region of C5a peptide" refers to the C-terminal 30% of the complement component C5a peptide.

As used herein, the term "wherein said antibody is not reactive with the C-terminal of C5a region" refers to antibodies that do not recognize or bind to the C-terminal 30% of the C5a peptide.

As used herein, the term "C-terminal truncated C5a peptides" refers to peptides of varying lengths derived from the N-terminal 70% of the C5a peptide, which do not include amino acid sequences from the C-terminal 30% of the C5a peptide. Examples of these peptides include, but are not limited to, SEQ ID NO:2 (from Rat C5a peptide), and SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:14, SEQ ID NO:15, and SEQ ID NO:16 (from Human C5a peptide).

As used herein, the terms "C5a peptide", "C5a protein", and "complement component C5a peptide" all refer to the complement component peptide in animals which is cleaved from the amino terminus of complement component C5 when the complement system is activated. Examples of animals with this protein include, but are not limited to, mice, rats, cows, pigs, and humans. This definition also includes peptides with synthetic sequences which share substantial homology to naturally occurring C5a peptides. An example of this type of sequence, includes, but is not limited to, the sequence disclosed in Mandecki W, *et al.*, *Proc Natl Acad Sci* U S A. Jun;82(11):3543- 7(1985).

As used herein, the term "modifying the amino acid sequence" of said C-terminal truncated C5a peptide refers to the addition, deletion, or substitution of one or more amino acids to create a variant or modified C-terminal truncated C5a peptide (*See* section II.b, below). Examples of such variants or modified sequences are listed in Table 3 below.

A "variant" of a C5a peptide (or C-terminal truncated C5a peptide) is defined as an amino acid sequence which differs by one or more amino acids from the C5a peptide (or C-terminal truncated C5a peptide) sequence. The variant may have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties, *e.g.* replacement of leucine with isoleucine. More rarely, a variant may have "nonconservative" changes, *e.g.* replacement of glycine with a tryptophan. Similar minor variations may also include amino acid deletions or insertions (*i.e.*, additions), or both. A variant may be an epitope as short as four amino acids in length, and as long as a modified full-lenth C5a peptide. More preferably, a variant is greater than five amino acids in length, and less than twenty-five amino acids in length. Variants may also contain a fusion protein. In such cases, the variant may have more amino acids than the natural, full-lenght C5a peptide.

DESCRIPTION OF THE INVENTION

The present invention relates to compositions and methods for the prevention treatment of blood-borne and toxin mediated diseases, and in particular anti-C5a antibodies for the prevention and treatment of sepsis caused by various types of organisms in humans as well as other animals. It is contemplated that the present invention finds use in the treatment of gram-negative and gram-positive sepsis. Although the invention may be used for treatment of sepsis due to an individual organism, it may also be used to treat sepsis caused by multiple organisms (e.g., sepsis and/or bacteremia due to gram-negative and gram-positive organisms).

I. The C5a Peptide and Sepsis

The complement system is a complex group of proteins present in body fluids that, working together with antibodies or other factors, plays an important role as mediators of immune, allergic, immunochemical and immunopathological reactions. Activation of the complement system can result in a wide range of reactions such as lysis of various kinds of cells, bacteria and protozoa, inactivation of viruses, and the direct mediation of inflammatory processes. Through the hormone-like activity of several of its components, the complement system can recruit and enlist the participation of other humoral and cellular effector systems. These in turn can induce directed migration of leukocytes, trigger histamine release from mast cells, and stimulate the release of lysosomal constituents from phagocytes.

The complement system consists of at least twenty distinct plasma proteins capable of interacting with each other, with antibodies, and with cell membranes. Many of these proteins, when activated, combine with still others to form enzymes that cleave and activate still other proteins in the system. The sequential activation of these proteins follows two main pathways, the classical pathway and the alternative pathway. Both pathways use a common terminal trunk that leads to cell lysis or virus inactivation.

The classical pathway can be activated by antigen-antibody complexes, aggregated immunoglobulins and non-immunological substances such as DNA and

trypsin-like enzymes. The classical pathway includes activation of C1, C4, C2 and C3. These components can be grouped into two functional units: C1 or recognition unit; and C4, C2 and C3 or activation unit. Five additional components denominated C5, C6, C7, C8, and C9 define the membrane attack unit forming the terminal trunk common to both pathways.

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C5a peptide, also called anaphylatoxin, is a complement component peptide which is cleaved from the amino terminus of component C5 when the complement system is activated. C5a peptide has been shown to stimulate contraction of smooth muscle, enhance vascular permeability, promote the synthesis and release of other mediators including leukotrienes, prostaglandins, platelet-activating factor, and histamine. *In vivo*, C5a peptide results in the accumulation of polymorphonuclear leukocytes (PMN) (*i.e.* neutrophils) and marcrophages at the site of inflammation, one of the hallmark events of an acute inflammatory response. *In vitro*, C5a peptide is a potent chemotaxin for leukocytes, most notably PMN and macrophages, and it activates PMN causing them to release a variety of hydrolytic enzymes and to generate oxygen radicals. These latter phenomena are thought to be responsible not only for the killing of microorganisms but for much of the tissue destruction that takes place in inflammatory situations.

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There is abundant evidence that in sepsis, complement activation, production of cytokines, and unregulated inflammatory responses occurs. It is well established in humans with sepsis that complement activation and complement consumption have occurred, as defined by loss of whole hemolytic activity of serum complement (CH50) and the presence of C5a peptide in serum [Koehl, J., Bitter-Suermann, D., *Anaphylatoxins. Complement in health and disease.*, Edited by Whaley, K., Loos, M., Weiler, J.M., Kluwer Academic publishers, pp 299-324, (1993), and Solomkin, *et al.*, *Surgery* 90:319-327, (1981)].

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It is well established from *in vitro* studies that interaction of C5a peptide with C5a receptor (C5aR) leads to phosphorylation of serine residues of the receptor, followed by rapid internalization of the receptor-ligand complex, dephosphorylation of

the receiver and its recycling back to the surface of the cell. All of this occurs fairly rapidly. Furthermore, the maximal C5a-induced H_2O_2 response of the neutrophil requires that only a fraction of C5aR be occupied with ligand [Van Epps, *et al.*, *J. Immunol.* 150:246-252 (1993)]. Neutrophils stimulated with C5a peptide become refractory ("deactivated") to further stimulation with this peptide; following exposure to high doses of C5a peptide, global deactivation to chemotactic peptides occurs [Ward, P.A. & Becker, E.L., *J. Exp. Med.* 127:693-709 (1968)]. There is clinical evidence that blood neutrophils from humans with early sepsis lose functional responsiveness to C5a peptide and in the latter phases of sepsis lose responsiveness to structurally different chemotaxins such as the bacterial chemotactic factor [Solomkin, J.S., *et al.*, *Surgery* 90:319-327 (1981)]. It has also been reported that C5 deficient mice demonstrate somewhat prolonged survival times when sepsis is induced, but ultimately all animals succumbed to the sepsis syndrome [Olson, L.M., *et al.*, *Ann. Surg.* 202:771-776 (1985)].

It is not necessary to the successful practice the present invention that one understand the precise mechanism by which a therapeutic benefit is achieved, nor is the present invention limited to any particular mechanistic explanation. However, it is believed that sepsis results in excessive production of C5a peptides, which leads to deactivation of neutrophils, compromising the respiratory burst (H_2O_2 production) of these cells and the closely linked bactericidal function, which is dependent upon H_2O_2 generation and participation of myeloperoxidase. The anti-C5a antibodies of the present invention, therefore, are believed to prevent the deactivation of neutrophils caused by sepsis, thus preserving the bactercidial function of the neutrophils. In this regard, the present invention contemplates antibodies specific for complement component C5a peptides and methods of using these antibodies to treat sepsis. In some embodiments, these antibodies are specific for complement component C5a peptide, wherein said C5a peptide has a C-terminal region and an N-terminal region, and wherein said antibody is not reactive with said C-terminal region.

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II. Generating Antibodies to C5a Peptides

a. Antibodies

The present invention contemplates monoclonal, polyclonal, and humanized antibodies to C5a peptides. In some embodiments, the antibodies are specific for complement component C5a peptide, wherein said C5a peptide has a C-terminal region and an N-terminal region, and said antibody is not reactive with said C-terminal region.

Monoclonal antibodies useful in this invention are obtained, for example, by well known hybridoma methods. In one embodiment, an animal is immunized with a preparation containing C-terminal truncated peptides. A fused cell hybrid is then formed between antibody-producing cells from the immunized animal and an immortalizing cell such as a myeloma. In one embodiment, antibodies of the present invention are produced by murine hybridomas formed by fusion of mouse myeloma or hybridoma which does not secrete antibody with murine spleen cells which secrete antibodies obtained from mice immunized against C-terminal truncated C5a peptides.

In some embodiments, mice are immunized with a primary injection of C-terminal truncated C5a peptides, followed by a number of boosting injections. During or after the immunization procedure, sera of the mice may be screened to identify mice in which a substantial immune response to the C-terminal truncated C5a peptides has been evoked. From the selected mice, spleen cells are obtained and fusions are performed. Suitable fusion techniques include, but are not limited to, the Sendai virus technique [Kohler, G. and Milstein, C., *Nature* 256:495 (1975)] or the polyethylene glycol method [Kennet, R.H., "*Monoclonal Antibodies, Hybridoma--A New Dimension in Biological Analysis*," Plenum Press, NY (1980)].

The hybridomas are then screened for production of anti-C5a antibodies. Suitable screening techniques include, but are not limited to, solid phase radioimmunoassay. A solid phase immunoadsorbent is prepared by coupling C5a peptides to an insoluble matrix. The immunoadsorbent is brought into contact with culture supernatants of hybridomas. After a period of incubation, the solid phase is separated from the supernatants, then contacted with a labelled antibody against murine

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immunoglobulin. Label associated with the immunoadsorbent indicates the presence of hybridoma products reactive with C5a peptides.

In preferred embodiments the monoclonal anti-C5a antibodies are produced in large quantities by injecting anti-C5a antibody producing hybridoma cells into the peritoneal cavity of mice and, after an appropriate time, harvesting acites fluid from the mice which yield a high titer of homogenous antibody. The monoclonal antibodies are isolated therefrom. Alternatively, the antibodies are produced by culturing anti-C5a antibody producing cells *in vitro* and isolating secreted monoclonal anti-C5a antibodies from the cell culture medium directly.

Another method of forming antibody-producing cells is by viral or oncogenic transformation. For example, a B-lymphocyte which produces anti-C5a specific antibody is infected and transformed with a virus, such as the Epstein-Barr virus, to give an immortal antibody-producing cell [Kozbon and Roder, *Immunol. Today* 4:72-79 (1983)].

The present invention also contemplates anti-C5a polyclonal antibodies. Polyclonal antibodies can be prepared by immunizing an animal with a crude preparation of C-terminal truncated C5a peptides, or purified C-terminal truncated C5a peptides. The animal is maintained under conditions whereby antibodies reactive with the components of the peptides are produced. [*See e.g.* Elzaim, *et al., Infect. Immun.* May;66(5):2170-9 (1998)]. Typically the animal is "boosted" by additional immunizations to increase the antibody titer. In one method, blood is collected from the animal upon reaching a desired titer of antibodies. The serum containing the polyclonal antibodies (antisera) is separated from the other blood components. The polyclonal antibody-containing serum may be further separated into fractions of particular types of antibodies (*e.g.* IgG or IgM) or monospecific antibodies can be affinity purified from polyclonal antibody containing serum. In another method, the immunized animal is a bird. In this method antibodies (IgY) are collected from egg yolks. The egg yolk is separated from the yolk lipid and non-antibody proteinaceous

matter, recovering the IgY anti-C5a antibodies in purified form (*see e.g.* U.S. Pat. 4,357,272 to Polson and U.S. Pat. 5,904,922 to Carroll).

The present invention also contemplates humanized antibodies (*i.e.* substantially non-immunogenic antibodies). Such antibodies are particularly useful in treating

5   human subjects. Chimeric and 'reshaped' humanized anti-C5a antibodies may be produced according to techniques known in the art (*see e.g.* U.S. Pat. 5,585,089 to Queen *et al.*, and Kettleborough, *et al., Protein Engineering*, vol. 4, no.7, pp 773-783, 1991). In one embodiment, humanized anti-C5a chimeric antiboides are produced using a combinatorial approach (*see e.g.* U.S. Pat. 5,565,332 to Hoogenboom *et al.*

10   and U.S. Pat. 5,658,727 to Barbas *et al.*). The present invention also contemplates single polypeptide chain binding molecules which have binding specificity and affinity subtantially similar to the binding specificity and affinity of the light and heavy chain aggregate variable region of an anti-C5a antibody (*see e.g.* U.S. Pat. 5,260,203 to Ladner *et al.*).

15   **b. C5a Peptide Immunogens**

The present invention provides various C5a peptide immunogens. For example, the C5a peptides can be from various animals (*e.g.* human, rat, pig, and cow). The amino acid sequence of these C5a peptides are described in the literature [*see* Rothermel *et al., Biochim. Biophys. Acta* 1351 (1-2), 9-12, (1997){rat}; Babkina, I.N.,

20   *et al., Bioorg Khim*, May;21(5):359-64, (1995){human}; Gerard, C. *et al., J. Biol. Chem.* 255(10), 4710-4715, (1980){pig}; and Zarbock, J., *et al., FEBS Lett.* 238(2), 289-294, (1988){cow}]. The C5a immunogen may be the full length C5a peptide, or various peptides derived from the full length C5a peptide. In particular embodiments, the peptides are C-terminal truncated peptides (*e.g.* SEQ ID NOS:2, 4, 5, 14, 15 and

25   16). Representative sequences are listed in Table 1. Representative human and rat DNA sequences which are used to generate various C-terminal truncated C5a peptides are listed in Table 2, along with the full human and full rat C5a DNA sequences. Modifications of these sequences (*i.e.* longer/shorter sequence, from various regions)

are contemplated by the present invention. Generation of these various C-terminal truncated C5a peptide immunogens are described below.

Variants of the C-terminal truncated C5a peptides are contemplated as useful immunogens (*See e.g.* Table 3). For example, it is contemplated that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid (*i.e.*, conservative mutations) will not have a major effect on the biological activity of the resulting molecule. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids can be divided into four families: (1) acidic (aspartate, glutamate); (2) basic (lysine, arginine, histidine); (3) nonpolar (alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan); and (4) uncharged polar (glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine). Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. In similar fashion, the amino acid repertoire can be grouped as (1) acidic (aspartate, glutamate); (2) basic (lysine, arginine histidine), (3) aliphatic (glycine, alanine, valine, leucine, isoleucine, serine, threonine), with serine and threonine optionally be grouped separately as aliphatic-hydroxyl; (4) aromatic (phenylalanine, tyrosine, tryptophan); (5) amide (asparagine, glutamine); and (6) sulfur -containing (cysteine and methionine) (*See e.g.*, Stryer ed., *Biochemistry*, 2nd ed,, WH Freeman and Co.[1981]).

Thus, in certain embodiments, modifications of the C-terminal truncated C5a peptides are contemplated by the present invention. Similar minor variations may also include amino acid deletions or insertions (*i.e.* additions), or both. Guidance in determining which and how many amino acid residues may be substituted, inserted or deleted without abolishing biological or immunological activity may be found using computer programs well known in the art, for example, DNAStar software or GCG (Univ. of Wisconsin).

Whether a change in the amino acid sequence of a C-terminal truncated C5a peptide results in a useful immunogen for producing the anti-C5a antibodies of the

present invention can be readily determined. One method involves screening the C-terminal truncated C5a peptides for the ability to inhibit the chemotaxis of neutrophils. Useful immunogens are identified by the ability to induce chemotaxis (*See e.g.* Examples 7, 8 and 11). Another indication of a useful immunogen is the ability of the

5   C-terminal truncated C5a peptide to inhibit chemotaxis when combined with C5a peptide (*See e.g.* Examples 7, 8, and 11). Another method involves screening the C-terminal truncated C5a peptides for the ability to antagonize the binding of labelled C5a peptides to neutrophils in a competitive assay (*See e.g.* Examples 10 and 11). Yet another method involves administering the C-terminal truncated C5a peptides to CLP

10   sepsis induced rats, and monitoring their response over a given time period. Useful immunogens are identified by the ability to reduce the symptoms of sepsis, and/or increase survival times of the rats (*See e.g.* Example 11).

The C-terminal truncated C5a peptides employed in the present invention may also comprise a fusion partner. Examples of fusion partners include Protein A, ABP,

15   GST, poly histidine, HA, KLH, and MBP. Other fusion partners are well known in the art. (*See* Nilsson *et al.*, *Prot. Expr. Purif.*, 11(1):1-16 [1997]). The fusion partner may serve various functions, including, but not limited to, enhancement of the solubility of the C-terminal truncated C5a peptides, as well as providing an "affinity tag" to allow the purification of the recombinant fusion C-terminal truncated C5a

20   peptide from the host cell or culture supernatant, or both. If desired, the exogenous protein fragment may be removed from the peptide of interest prior to immunization by a variety of enzymatic or chemical means known in the art.

In some embodiments, nucleic acid sequences corresponding to these various C-terminal truncated C5a peptides (*e.g.*, SEQ ID NOS:10, 11, 13, 17, 18, and 19) are

25   used to generate recombinant DNA molecules that direct the expression of the C-terminal truncated C5a peptides in appropriate host cells, which are then purified and used as immunogens to generate the antibodies of the present invention. These DNA sequences may be included in any one of a variety of expression vectors for expressing C-terminal truncated C5a peptides in various hosts. Examples of vectors include, but

are not limited to, chromosomal, nonchromosomal and synthetic DNA sequences, *e.g.*, derivatives of SV40; bacterial plasmids; phage DNA; baculovirus; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies, and the like. Any vector may be used as long as it is replicable and viable in the host.

Large numbers of suitable vectors are known to those of skill in the art, and are commercially available. Such vectors include, but are not limited to, the following: 1) Bacterial - pQE70, pQE60, pQE-9 (Qiagen), pBS, pD10, phagescript, psiX174, pbluescript SK, pBSKS, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene); ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia); and 2) Eukaryotic - pWLNEO, pSV2CAT, pOG44, PXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, pSVL (Pharmacia). In general, mammalian expression vectors comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

The DNA sequence in the expression vector may be operably linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. Promoters useful in the present invention include, but are not limited to, the LTR or SV40 promoter, the *E. coli. lac* or *trp*, the phage lambda PL and PR, T3 and T7 promoters, and the CMV immediate early, HSV thymidine kinase, and mouse metallothionein-I promoters and other promoters known to control expression of peptides in prokaryotic or eukaryotic cells or their viruses. Recombinant expression vectors generally include origins of replication and selectable markers permitting transformation of the host cell (*e.g.*, dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in *E. coli*).

Transcription of the DNA encoding the C-terminal truncated C5a peptides of the present invention is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act

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on a promoter to increase its transcription. Enhancers useful in the present invention include, but are not limited to, the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. The expression vector may also contain a ribosome binding site for translation initiation and a transcription terminator.

Various host cells may be employed to recombinantly express the C-terminal truncated C5a peptides. Suitable host cells are higher eukaryotic cells (*e.g.*, a mammalian or insect cell), lower eukaryotic cells (*e.g.*, a yeast cell), and prokaryotic cells (*e.g.*, a bacterial cell). Specific examples of host cells include, but are not limited to, *Escherichia coli, Salmonella typhimurium, Bacillus subtilis*, and various species within the genera *Pseudomonas, Streptomyces, and Staphylococcus*, as well as, *Saccharomycees cerivisiae, Schizosaccharomycees pombe, Drosophila* S2 cells, *Spodoptera* Sf9 cells, Chinese Hamster Ovary (CHO) cells, COS-7 lines of monkey kidney fibroblasts, (Gluzman, Cell, 23:175 [1981]), C127, 3T3, HeLa and BHK cell lines.

The constructs in host cells can be used in a conventional manner to produce the C-terminal truncated C5a peptides encoded by the recombinant sequences. In some embodiments, introduction of the construct into the host cell is effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation [Davis *et al., Basic Methods in Molecular Biology*, (1986)].

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (*e.g.*, temperature shift or chemical induction) and cells are cultured for an additional period. Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Methods for recovering and purifying C-terminal truncated C5a peptides from recombinant cell culture include, but are not limited to, ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography,

phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. High performance liquid chromatography (HPLC) can also be employed for final purification steps.

DNA sequences having coding sequences (*e.g.*, SEQ ID NOS:10, 11, 13, 17, 18, and 19) fused in frame to a marker sequence allow for purification of the C-terminal truncated C5a peptide. One example is a histidine tag which may be supplied by a vector (*e.g.*, a pQE-9 vector) which provides for purification of the polypeptide fused to the marker in the case of a bacterial host, or, for example, the marker sequence may be a hemagglutinin (HA) tag when a mammalian host (*e.g.*, COS-7 cells) is used. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson, *et al.*, *Cell*, 37:767 [1984]).

The coding sequences for the C-terminal truncated C5a peptides can also be incorporated as a part of a fusion gene including a nucleotide sequence encoding a different polypeptide. One example employs the VP6 capsid protein of rotavirus used as an immunologic carrier protein for the C-terminal truncated C5a peptides, either in the monomeric form or in the form of a viral particle. The nucleic acid sequences corresponding to the various C5a peptides to which antibodies are to be raised can be incorporated into a fusion gene construct which includes coding sequences for a late vaccinia virus structural protein to produce a set of recombinant viruses expressing fusion proteins comprising C-terminal truncated C5a peptides as part of the virion. It has been demonstrated with the use of immunogenic fusion proteins utilizing the Hepatitis B surface antigen fusion proteins that recombinant Hepatitis B virions can be utilized in this role as well. Similarly chimeric constructs coding for fusion proteins containing C-terminal truncated C5a peptides and the poliovirus capsid protein are created to enhance immunogenicity of the set of polypeptide antigens [*see e.g.*, EP Publication No. 025949; and Evans *et al.*, *Nature* 339:385 (1989); Huang *et al.*, *J. Virol.* 62:3855 (1988); and Schlienger *et al.*, *J. Virol.* 66:2 (1992)]. The Multiple Antigen Peptide system for peptide-based immunization can also be utilized, wherein a

desired C-terminal truncated C5a peptide sequence is obtained directly from organo-chemical synthesis of the peptide onto an oligomeric branching lysine core [*see e.g.*, Posnett *et al., JBC* 263:1719 (1988) and Nardelli *et al., J. Immunol.* 148:914 (1992)]. C-terminal truncated C5a peptides can also be expressed and presented by

5   bacterial cells in order to generate the antibodies of the present invention.

In addition to utilizing fusion proteins to enhance immunogenicity, fusion proteins can also facilitate the purification of proteins. Accordingly, the C5a peptides can be generated as a glutathione-S-transferase (GST) fusion protein. Such GST fusion proteins enable easy purification of the C5a peptides, such as by the use of

10   glutathione-derivatized matrices [*see e.g, Current Protocols in Molecular Biology*, Eds. Ausabel *et al.*, N.Y.: John Wiley & Sons, (1991)]. Also, a fusion gene coding for a purification leader sequence, such as a poly-(His)/enterokinase cleavage site sequence at the N-terminus of the desired C-terminal truncated C5a peptide, can allow purification of the expressed C-terminal truncated C5a fusion protein by affinity

15   chromatography using, for example, a Ni2+ metal resin. The purification leader sequence can then be subsequently removed by treatment with enterokinase [*see e.g.*, Hochuli *et al., J. Chromatography* 411:177 (1987)].

Techniques for making fusion genes are well known. Essentially, the joining of various DNA fragments coding for different polypeptide sequences is performed in

20   accordance with conventional molecular biology techniques, employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. The fusion gene is synthesized by conventional techniques including automated DNA synthesizers.

25   Alternatively, in other embodiments of the present invention, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which is subsequently annealed to generate a chimeric gene sequence [*see e.g., Current Protocols in Molecular Biology*, Eds. Ausubel *et al.*, John Wiley & Sons (1992)].

The C-terminal truncated C5a peptide sequences may be synthesized, whole or in part, using chemical methods well known in the art [see e.g., Caruthers et al., Nuc. Acids Res. Symp. Ser. 7:215-233 (1980); Crea and Horn, Nuc. Acids Res. 9:2331 (1980); Matteucci and Caruthers, Tetrahedron Lett 21:719 (1980); and Chow and Kempe, Nuc. Acids Res. 9:2807-2817 (1981)]. For example, C-terminal truncated C5a peptides can be synthesized by solid phase techniques, cleaved from the resin, and purified by preparative high performance liquid chromatography [see e.g., Creighton (1983) Proteins Structures And Molecular Principles, W H Freeman and Co, New York N.Y.]. In other embodiments of the present invention, the composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (see e.g., Creighton, supra).

Direct peptide synthesis can be performed using various solid-phase techniques [Roberge et al., Science 269:202-204 (1995)] and automated synthesis may be achieved, for example, using ABI 431A Peptide Synthesizer (Perkin Elmer, Norwalk Conn.) in accordance with the instructions provided by the manufacturer. Additionally the amino acid sequence of the C-terminal truncated C5a peptide sequences may be altered during direct synthesis yielding various C-terminal truncated C5a peptides which are used to generate the anti-C5a antibodies of the present invention.

Compounds mimicking the necessary conformation for generating the antibodies of the present invention are also contemplated as within the scope of the invention. For example, mimetics of the C-terminal truncated C5a peptides are contemplated. A variety of designs for such mimetics are possible. For example, cyclic C-terminal truncated C5a peptides, in which the necessary conformation for immunogenicity is stabilized by non-peptides, are specifically contemplated (See e.g. U.S. Pat. 5,192,746 to Lobl, et al., U.S. Pat. 5,169,862 to Burke, Jr., et al., U.S. Pat. 5,539,085 to Bischoff, et al., U.S. Pat. 5,576,423 to Aversa et al., U.S. Pat. 5,051,448 to Shashoua, and U.S. Pat. 5,559,103 to Gaeta et al., all of which describe muliple methods for creating such compounds). The present invention also contemplates non

peptide compounds that mimic C-terminal truncated C5a peptides, as well as multimeric compounds that repeat the relevant peptide sequences.

Table 1

C5a Sequences and Peptides Useful in Generating Antibodies

Species	SEQ ID NO:	Amino Acid Sequence
Rat C5a (full seq.)	SEQ ID NO:1	DLQLLHQKVEEQAAKYKHRVP KKCCYDGARENKYETCEQRVA RVTIGPHCIRAFNECCTIADKIR KESHHKGMLLGR
Rat C5a (residues 17-36)	SEQ ID NO:2	KHRVPKKCCYDGARENKYET
Human C5a (full seq.)	SEQ ID NO:3	MLQKKIEEIAAKYKHSVVKKCC YDGASVNNDETCEQRAARISLG PRCIKAFTECCVVASQLRANISH KDMQLGR
Human C5a (res. 1-20)	SEQ ID NO:4	MLQKKIEEIAAKYKHSVVKK
Human C5a (res. 21-40)	SEQ ID NO:5	CCYDGASVNNDETCEQRAAR
Human C5a (res. 55-74)	SEQ ID NO:6	CVVASQLRANISHKDMQLGR
Human C5a (res. 12-20)	SEQ ID NO:14	KYKHSVVKK
Human C5a (res. 28-33)	SEQ ID NO:15	VNNDET
Human C5a (res. 38-46)	SEQ ID NO:16	AARISLGPR
Bovine C5a (full seq.)	SEQ ID NO:7	MLKKKIEEEAAKYRNAWVKKC CYDGAHRNDDETCEERAARIAI GPECIKAFKSCCAIASQFRADEH HKNMQLGR
Porcine C5a (full seq.)	SEQ ID NO:8	MLQKKIEEEAAKYKYAMLKKC CYDGAYRNDDETCEERAARIKI GPKCVKAFKDCCYIANQVRAEQ SHKNIQLGR

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Rat C5a (res. 58-77)　　　　SEQ ID 75　　　CTIADKIRKESHHKGMLLGR

Table 2

Rat and Human C5a Polynucleotide Sequences Useful in Generating C5a Peptides

Species	SEQ ID NO:	Polynucleotide Sequence
Human C5a (full seq.)	SEQ ID NO:9	GATCCAGTATGTTGCAAAAAA AAATTGAAGAAATTGCTGCTA AATATAAACATTCTGTTGTTAA AAAATGTTGTTATGATGGAGCT TCTGTTAATAATGATGAAACCT GCGAACAACGCGCTGCTAGAA TTTCTTTGGGACCTAGATGTA TTAAAGCATTTACAGAATGTTG TGTTGTTGCTTCTCAATTGAG GCGAATATTTCTCATAAAGATA TGCAATTGGGAAGATAGGATC CGTCGA
Human C5a (for res. 1-20)	SEQ ID NO:10	ATGTTGCAAAAAAAAATTG AAGAAATTGCTGCTAAATA TAAACATTCTGTTGTTAAAAAA
Human C5a (for res. 21-40)	SEQ ID NO:11	TGTTGTTATGATGGAGCTTC TGTTAATAATGATGAAACCT GCGAACAACGCGCTGCTAGA
Human C5a (for res. 12-20)	SEQ ID NO:17	TTGCTGCTAAATATAAACAT TCTGTTG
Human C5a (for res. 28-33)	SEQ ID NO:18	GAGCTTCTGTTAATAATG
Human C5a (for res. 38-46)	SEQ ID NO:19	AACAACGCGCTGCTAGAATT TCTTTGG

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Rat C5a (full seq.)	SEQ ID NO:12	GACCTGCAGCTCCTGCATCAG
		AAAGTGGAAGAACAAGCTGCT
		AAATACAAACACCGTGTGCCC
		AAGAAATGCTGTTATGATGGA
		GCCCGAGAAAACAAATACGAA
		ACCTGTGAGCAGCGAGTTGCC
		CGGGTGACCATAGGCCCACAC
		TGCATCAGGGCCTTCAACGAG
		TGTTGTACTATTGCGGATAAG
		ATCCGAAAAGAAAGCCACCAC
		AAAGGCATGCTGTTGGGAAGG
Rat C5a (for res. 17-36)	SEQ ID NO:13	AAACACCGTGTGCCCAAGAAA
		TGCTGTTATGATGGAGCCCGA
		GAAAACAAATACGAAACC
Rat C5a (res. 58-77)	SEQ ID NO:76	TGTACTATTGCGGATAAG
		ATCCGAAAAGAAAGCCACCAC
		AAAGGCATGCTGTTGGGAAGG

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Table 3

Variant C-Terminal Truncated C5a Peptide Sequences

SEQ ID NO:	Amino Acid Sequence
SEQ ID NO:20	KYKHTVVKK
SEQ ID NO:21	KYKHSAVKK
SEQ ID NO:22	KYKHSAAKK
SEQ ID NO:23	KYKHSVAKK
SEQ ID NO:24	VNNQET
SEQ ID NO:25	VNNDES
SEQ ID NO:26	VNNQES
SEQ ID NO:27	ANNDET
SEQ ID NO:28	AARISIGPR
SEQ ID NO:29	AARISVGPR
SEQ ID NO:30	AARITLGPR
SEQ ID NO:31	AVRISLGPR
SEQ ID NO:32	VARISLGPR
SEQ ID NO:33	VVRISLGPR
SEQ ID NO:34	MLQKKIEEIAAKYKHSVVK
SEQ ID NO:35	MLQKKIEEIAAKYKHSVV
SEQ ID NO:36	MLQKKIEEIAAKYKHSV
SEQ ID NO:37	MLQKKIEEIAAKYKHS
SEQ ID NO:38	MLQKKIEEIAAKYKH
SEQ ID NO:39	LQKKIEEIAAKYKHSVVKK
SEQ ID NO:40	QKKIEEIAAKYKHSVVKK
SEQ ID NO:41	KKIEEIAAKYKHSVVKK
SEQ ID NO:42	KIEEIAAKYKHSVVKK
SEQ ID NO:43	IEEIAAKYKHSVVKK
SEQ ID NO:44	MIQKKIEEIAAKYKHSVVKK
SEQ ID NO:45	MVQKKIEEIAAKYKHSVVKK

SEQ ID NO:46	MLDKKIEEIAAKYKHSVVKK
SEQ ID NO:47	MLQKKIEEIAAKYKHTVVKK
SEQ ID NO:48	MLQKKIEEIVAKYKHSVVKK
SEQ ID NO:49	MLQKKIEEIVVKYKHSVVKK
SEQ ID NO:50	MLQKKIEEIAAKYKHSVAKK
SEQ ID NO:51	MLQKKIEEIAAKYKHSAAKK
SEQ ID NO:52	MLQKKIEEIAAKYKHSAVKK
SEQ ID NO:53	MLQKKIEEIAVKYKHSVVKK
SEQ ID NO:54	CCYDGASVNNDETCEQRAA
SEQ ID NO:55	CCYDGASVNNDETCEQRA
SEQ ID NO:56	CCYDGASVNNDETCEQR
SEQ ID NO:57	CCYDGASVNNDETCEQ
SEQ ID NO:58	CCYDGASVNNDETCE
SEQ ID NO:59	CYDGASVNNDETCEQRAAR
SEQ ID NO:60	YDGASVNNDETCEQRAAR
SEQ ID NO:61	DGASVNNDETCEQRAAR
SEQ ID NO:62	GASVNNDETCEQRAAR
SEQ ID NO:63	ASVNNDETCEQRAAR
SEQ ID NO:64	CCYQGASVNNDETCEQRAAR
SEQ ID NO:65	CCYDGASVNNQETCEQRAAR
SEQ ID NO:66	CCYQGASVNNQETCEQRAAR
SEQ ID NO:67	CCYDGASVNNDESCEQRAAR
SEQ ID NO:68	CCYDGATVNNDETCEQRAAR
SEQ ID NO:69	CCYDGVSVNNDETCEQRAAR
SEQ ID NO:70	CCYDGASANNDETCEQRAAR
SEQ ID NO:71	CCYDGASVNNDETCEQRVAR
SEQ ID NO:72	CCYDGASVNNDETCEQRVVR
SEQ ID NO:73	CCYDGASVNNDETCEQRAVR
SEQ ID NO:74	CCYDGVSANNDETCEQRVVR

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In one embodiment, the present invention contemplates a method of producing polyclonal antibodies, comprising; providing an animal and an immunogenic composition, wherein the composition comprises C-terminal truncated C5a peptides; and immunizing the animal with the immunogenic composition in order to generate antibodies. It is not intended that the present invention be limited to particular C-terminal truncated peptides. A variety of C-terminal truncated peptides are contemplated. In one embodiment, the C-terminal truncated peptide corresponds to the entire N-terminal region of C5a peptide. In another embodiment, the C-terminal truncated peptide is a fragment or portion of the N-terminal region of C5a peptide. In another embodiment, the fragment or portion of the N-terminal region of C5a peptide is between approximately 5 and approximately 50 amino acids in length. In some embodiments, the C-terminal truncated peptide is fifty amino acids in length. In other embodiments, the C-terminal truncated peptides are approximately five amino acids in length. In preferred embodiments, the C-terminal truncated peptides are approximately 20 amino acids in length. In especially preferred embodiments, the C-terminal truncated peptides are selected from SEQ ID NOS:2, 4, and 5.

III. Antibody Applications

A. Prophylactic Use In Humans

The diagnosis of sepsis is problematic. First, the development of sepsis does not require the persistent release of toxin(s), nor the presence of organisms, in the circulation. Thus, many patients who die of sepsis are never shown to be bactermic. [R.C. Bone, *Ann. Intern. Med.* 115:457-469 (1991)]. Second, even if bacteria are detected, the amount of time needed for this detection is often too great to be practical.

For these reasons and others, the present invention contemplates the use of anti-C5a antibodies in humans prior to the onset of symptoms (*e.g.*, prophylactically). In particular, the present invention contemplates the use of anti-C5a antibodies as prophylactic treatment in patients at high risk for infection, as well as sepsis.

High risk patients include surgical patients (particularly the elderly), low birth weight infants, burn victims and trauma patients. Trauma patients are particularly

difficult to examine because of the multitude of invasive procedures that they have undergone. Trauma patients are also typically hooked up to a number of devices, including intravascular lines, mechanical ventilators and Foley catheters. While every attempt is made to change intravascular lines, this is frequently impossible because of

5   the extent of trauma and the lack of venous accessibility. [E.S. Caplan and N. Hoyt, *Am. J. Med.* 70:638-640 (1981)].

Most patients with multiple trauma have fever, as well as increased white cell counts due to the stress of the trauma itself. The classic indicators of infection, therefore, may or may not reflect an ongoing infection.

10 Because of this, current clinical practice involves treating patients with antibiotics only for specific indications, and for as short a period of time as possible. Generally, the average course for any documented infection is seven to ten days. Prophylactic antibiotics are used in only three instances: open fractures, penetrating abdominal injuries and penetrating facial injuries in which there is injury to the

15 respiratory mucosa. Even in these situations, antibiotics are used for only three to five days, depending on the injury.

Burn patients have many problems with respect to the diagnosis and therapy for infection. Since the magnitude of thermal injury is related to the level of trauma in a burn victim, this becomes even more of a problem with acute cases. It is reported that

20 septicemia appears in the blood cultures of burn patients almost four days after a septic state. [M. Meek *et al.*, *J. Burn Care Rehab.* 12:564-568 (1991)]. Consequently, therapy with the antibodies of the present invention is particularly appropriate immediately after the burn injury as a means of preventing a septic reaction. Furthermore, in severe cases, consideration should be given to the topical

25 administration of the antibodies of the present invention to prevent wound sepsis.

Finally, surgical patients also represent a risk group where the antibodies of the present invention can be used successfully. Current practice involves the prophylactic use of antibiotics in a very narrow category of cases (*e.g.*, elective colorectal procedures, cholecystectomy, hysterectomy and Caesarean sections). [R.L. Nichols in

30 *Decision Making in Surgical Sepsis*, B.C. Decker, Inc., Philadelphia, pp. 20-21

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(1991)]. One to two grams of a broad-spectrum antibiotic are administered intravenously at the induction of anesthesia. An additional dose may be given during an extensive procedure or post-operatively but prophylaxis beyond 24 hours is not indicated. Twenty-four hours of antibiotic prophylaxis is considered to be sufficient to control contamination. Continuance of antibiotic prophylaxis beyond 24 hours is an added expense, particularly when using an antibiotic with short serum and tissue half-lives. Most importantly, continuation of antibiotic prophylaxis also runs an excessive risk of drug toxicity and emergence of resistant strains. As such, the present invention contemplates the use of anti-C5a antibodies to help reduce the need for antibiotics, and reduce the risk of sepsis.

In this regard, the present invention contemplates a method comprising; providing; a subject at risk for sepsis, and a therapeutic composition comprising an antibody specific for complement component C5a peptide, and prophylactically administering said therapeutic composition to the subject. In some embodiments administering the composition prevents the onset of symptoms of sepsis.

B. Acute Therapy In Humans

The present invention also contemplates the use of anti-C5a antibodies in a therapeutic preparation for acute treatment. In this case, treatment involves administration of the antibodies after infection is detected and/or sepsis is suspected.

Evidence suggestive of infection includes the following: (1) core temperature higher than 38°C or lower than 35°C; (2) peripheral blood leukocyte count greater than $12 \times 10^9/L$ or less than $3 \times 10^9/L$ (not due to chemotherapy), or at least 20% immature forms; (3) growth of gram-negative organisms from a blood culture drawn within the preceding 48 hours; or (4) documented or suspected site of gram-negative infection.

A systemic septic reaction is characterized by at least one of the following: arterial hypotension (systolic blood pressure <90 mm Hg or an acute drop of 30 mm Hg); metabolic acidosis (base deficit >5 mEq/L); decreased systemic vascular resistance (systemic vascular resistance <800 dynes/s · cm^5); tachypnea (respiratory

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rate >20/min or ventilation >10 L/min if mechanically ventilated); or otherwise unexplained dysfunction of the kidney (urine output <30 ml/h), or lungs.

It must be stressed that the anti-C5a antibodies of the present invention should ideally be used prior to a systemic infection, if possible. For example, the antibodies are administered immediately after bacteremia or fungemia is detected. Similarly, antibodies can be administered where there is an obvious sign of infection at a particular site (e.g., wounds, sinusitis, meningitis, respiratory, gastrointestinal, or urinary tract infections, etc.).

Primary bacteremia is typically defined as two or more blood cultures with the same bacterial organism occurring in a patient with no other obvious site of infection. Sinusitis is diagnosed in a patient who has at least two of the following: purulent nasal discharge, roentgenographic evidence of sinusitis or purulent material aspirated from the sinuses.

The lower respiratory tract is a common site of infection. Pneumonia in the intubated patient is diagnosed in a patient when there is fever, leukocytosis and a Gram stain with many polymorphonuclear leukocytes. Pneumonia may also be diagnosed in a patient with a new infiltrate that has not cleared with intensive physical therapy (this last criterion helps rule out atelectasis).

The C5a peptide has been implicated in the pathogenesis of bacterial meningitis [Stahel, et al., J. Immunol. Jul. 15;159(2):861-9 (1997)]. As such, treatment of acute meningitis with the anti-C5a antibodies of the present invention is contemplated. Among the bacterial causes of meningitis, two gram-negative organisms (Neisseria meningitidis and Haemophilus influenzae), and one gram-positive organism (Streptococcus pneumoniae), are the major culprits. N. meningitidis is responsible for an estimated 24-25% of meningitis in children one month of age through 15 years; for adults, the figure is 10-35%. H. influenzae is responsible for an estimated 40-60% of meningitis cases in children one month of age through 15 years, while S. pneumoniae is responsible for 10-20% of meningitis cases in the same age group, as well as 30-50% of cases in adults (over 15 years). [W.K. Joklik et al. (eds.), Zinsser

Microbiology, 18th ed., p. 485, Appleton-Century-Crofts, Norwalk, CT (1984).] Other organisms such as *Streptococcus spp.* in groups A and B, *Staphylococcus aureus, Listeria monocytogenes*, and various gram-negative bacilli (*e.g.*, enterics such as *E. coli*) are responsible for sporadic cases. Untreated, bacterial meningitis is fatal in 70-100% of patients, and infected neonates may have motor or intellectual impairment related to their infection. [J.M. Slack and I.S. Snyder, *Bacteria and Human Disease*, pp. 128-133, Yearbook Medical Publishers (1978).]

The blood-brain barrier represents a significant obstacle to treatment of meningitis, especially prophylactically. As the barrier is designed to prevent invasion of organisms and uptake of compounds (*e.g.*, antimicrobials), intravenous antimicrobial administration is not always sufficient. For example, estimates provided in experimental studies indicate that drug concentrations in the cerebrospinal fluid and brain are approximately 1/200 to 1/500 of those in serum. [G.P. Youmans *et al.*, *Biologic and Clinical Basis of Infectious Diseases*, 3d ed., p. 553, W.B. Saunders Co., (1985).] Even with the inflammatory changes associated with an intensity characteristic of bacterial meningitis, passage of antimicrobials is hindered by the barrier. [*Id.*]

Endotoxemia due to the release of endotoxins from dividing organisms and the presence of endotoxin in the cerebrospinal fluid (CSF) present serious complications during sepsis and meningitis. Endotoxin is detectable in the plasma and CSF of patients with meningitis due to gram-negative bacteria. (Awad *et al.*, *supra* at 560.) Perhaps due to increased permeability of the bowel mucosa, endotoxin may also be found in the plasma of patients with meningitis due to gram-positive organisms (*e.g., Streptococcus pneumoniae*).

Ironically, release of endotoxin is aggravated by antimicrobial treatment. Indeed, it is believed that aggressive antibiotic treatment can be life-threatening. This is due to the increased burden of endotoxin present in the blood and CSF which results when a large number of organisms are simultaneously killed by the antibiotic. This increased endotoxin burden results in the pathology associated with fatal meningitis

and is a significant problem facing clinicians who must treat a seriously ill patient within the first few hours of disease.

Therefore, the present invention contemplates treating acute septic conditions with anti-C5a antibodies. It is contemplated that these antibodies be administered alone, or in combination with other therapeutic preparations. In preferred embodiments, the present invention provides a method comprising; providing; a subject suffering from symptoms of sepsis, a therapeutic composition comprising an antibody specific for complement component C5a peptide, and administering the therapeutic composition to the subject.

C. Veterinary Care

Septicemia and sepsis are by no means limited to human beings. Infection by gram-negative bacteria accounts for significant morbidity and mortality in neonatal livestock, such as calves. [D.D. Morris *et al.*, *Am. J. Vet. Res.* 47:2554-2565 (1986).] Interestingly, humoral immune status is again related to susceptibility to sepsis and this is largely dependent on passive transfer from colostrum. For this reason, in some embodiments the present invention contemplates determining the immune status of the animal prior to administration of the anti-C5a antibodies. This determination can be made by screening neonatal calves for total circulating serum immunoglobulin (*e.g.*, by ELISA).

Where the immune status is poor (*e.g.*, low total IgG levels), the antibodies of the present invention should be used prophylactically. Where the animal's immune status is healthy, use of the antibodies may be needed for acute therapy of gram-negative bacterial sepsis, which remains prevalent in neonatal calves even with high natural antibody levels.

The present invention contemplates the treatment of other animals as well. For example, among foals less than 10 days of age in critical distress, sepsis is the most serious problem. [A.M. Hoffman *et al.*, *J. Vet. Int. Med.* 6:89-95 (1992).] Symptoms highly indicative of sepsis risk include weakness, metabolic disturbance and dehydration. In one embodiment, the invention contemplates using antibodies for

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prophylactic treatment of foals less than 10 days of age having these indicators, or those at risk of infection.

While positive blood cultures are found in less than half of the cases, those animals found positive have a very poor chance of survival. The present invention therefore contemplates using anti-C5a antibodies for acute treatment of any animal with evidence of septicemia, with or without culture-proven cases.

IV. Therapeutic Preparations And Combinations

In some embodiments, the present invention contemplates using therapeutic compositions of soluble anti-C5a antibodies. It is not intended that the present invention be limited by the particular nature of the therapeutic composition. For example, such compositions can be provided together with physiologically tolerable liquids, gels, solid carriers, diluents, adjuvants and excipients (and combinations thereof). In addition, anti-C5a antibodies may be used together with other therapeutic agents, including other immunoglobulins or antibiotics.

As noted above, these therapeutic compositions can be administered to mammals for veterinary use, such as with domestic animals, and clinical use in humans in a manner similar to other therapeutic agents. In general, the dosage required for therapeutic efficacy varies according to the type of use and mode of administration, as well as the particularized requirements of individual hosts. The attending medical professional is capable of determining the therapeutically effective dosage based on the characteristics of the subject (*e.g.* gender, age, weight, etc.)

With respect to the mode of administration, in some embodiments the antibodies are administered intravenously, intra-muscularly, subcutaneously, intradermally, intraperitoneally, intrapleurally, intrathecally, or topically. In some embodiments, formulations for such administrations may comprise an effective amount of anti-C5a antibodies in sterile water or physiological saline.

On the other hand, formulations may contain such normally employed additives as binders, fillers, carriers, preservatives, stabilizing agents, emulsifiers, buffers and excipients as, for example, pharmaceutical grades of mannitol, lactose, starch,

magnesium stearate, sodium saccharin, cellulose, magnesium carbonate, and the like. These compositions typically contain 1%-95% of active ingredient, preferably 2%-70%.

The compositions are preferably prepared as injectables, either as liquid

5   solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared.

The antibodies of the present invention are often mixed with diluents or excipients which are compatible and physiologically tolerable. Suitable diluents and excipients are, for example, water, saline, dextrose, glycerol, or the like, and

10   combinations thereof. In addition, if desired, the compositions may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, stabilizing or pH buffering agents.

Where repeated administrations are required, it may be beneficial to first clear any anti-hapten antibodies by administering free antibiotic. This can then be followed

15   by administration of the anti-C5a antibodies of the present invention.

EXPERIMENTAL

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

20   In the experimental disclosure which follows, the following abbreviations apply: N (normal); M (molar); mM (millimolar); µM (micromolar); mol (moles); mmol (millimoles); µmol (micromoles); nmol (nanomoles); pmol (picomoles); g (grams); mg (milligrams); µg (micrograms); ng (nanograms); l or L (liters); ml (milliliters); µl (microliters); cm (centimeters); mm (millimeters); µm (micrometers);

25   nm (nanometers); °C (degrees Centigrade); Sigma (Sigma Chemical Co., St. Louis, MO).

EXAMPLE 1

Induction of Sepsis In Rats by Cecal Ligation Puncture

This Examples describes the induction of sepsis by Cecal Ligation Puncture (CLP) in Male Long-Evans specific pathogen-free rats (275-300 mg) obtained from

5   Harlan, Inc., Indianapolis, Indiana. Anesthesia was induced by intraperitoneal administration of ketamine (20 mg/100 mg body weight). Through a 2 cm abdominal midline incision the cecum was ligated below the ileocecal valve without obstructing the ileum or colon. The cecum was then subjected to a single "through and through" perforation with a 21-gauge needle. After repositioning the bowel, the abdominal

10   incision was closed in lagers with plain gut surgical suture 4-0 (Ethicon, Inc., Somerville, NJ) and metallic clips. Sham animals underwent the same procedure except for ligation and puncture of the cecum.

EXAMPLE 2

Preparation and Characterization of Anti-Rat C5a Antibodies

15   This Example describes the preparation and characterization of anti-rat C5a antibodies. Rat C5a peptide with the sequence KHRVPKKCCYDGARENKYET (SEQ ID NO:2) was obtained from Research Genetics (Huntsville Ala.) and coupled to keyhole limpet hemocyanin (KLH). This 20-mer rat C-terminal truncated C5a peptide corresponds to amino acid residues 17-36 of the full length rat C5a peptide

20   [Rothermel, E. *et al.*, *Biochimica. et Biophysica. Acta.* 1351:9-12, (1997)], which is listed as SEQ ID NO:1. The coupled peptide was used as an antigen to immunize rabbits. After several injections, the antibody was affinity purified using the synthetic 20-mer peptide coupled to beads. Immunoprecipitation with activated rat serum using beads coupled with this antibody yielded a single band with a 14 kDa position in

25   Western blot analysis, characteristic of rat C5a peptide [*See*, Ward, P.A. and Becker, E.L., *J. Exp. Med.* 127:693-709 (1968)].

When added at a concentration of 100 µg/ml, the antibody did not affect whole hemolytic complement activity (CH50) of fresh rat serum. Addition of 0, 10, 20, 40, 80 micrograms of anti-C5a Ab yielded CH50 values of 99, 94, 91, 93, and 94

units/ml. Therefore, the fact that CH50 levels are not affected by the anti-C5a antibodies indicates that the anti-C5a antibodies are effective without compromising the entire complement system (including the classical and alternate pathways).

EXAMPLE 3

Anti-Rat C5a Antibodies Prevent Sepsis

This Example describes the treatment of sepsis. CLP-induced sepsis was generated in three different groups of rats according to the procedure of Example 1 above. The first group (n=21) was treated intravenously with 400 μg of preimmune IgG (in a volume of 300 μg) immediately after the CLP procedure. The second group (n=10) was treated intravenously with 400 μg of anti-C5a IgG prepared according to Example 2 above (in a volume of 300 μg) immediately after the CLP procedure. The third group (n=12) was depleted of C3 by four intraperitoneal injections of purified cobra venom factor (25 units per injection at 12 hour intervals) during the 48 hour period prior to induction of CLP. This protocol has been shown to reduce serum C3 levels to less than 3% of normal and to suppress whole complement hemolytic activity (CH50) to undetectable levels [Hill, J.H. and Ward, P.A., *J. Exp. Med.* 133:885-900, (1971)].

Survival rates for the three groups of rats were determined over a ten-day period, with assessment every 6 hours. A sham operated group of rats (n=10) in which no cecal puncture was performed, were also included in this study and all survived during the 10 day period.

All animals had unlimited access to food and water, both pre- and postoperatively. Survival data for the three CLP treated groups is depicted in Figure 1. In the CLP group receiving preimmune IgG, survival was 66.7% (14/21) 24 hours after CLP, diminishing progressively each day until day 8, at which time only 9.5% (2/21) were alive. In the C3 depleted group, the survival time was greatly reduced, with all animals dead before day 4.5. When this group was compared to the CLP group receiving preimmune IgG, the outcomes were statistically different (p=0.01 by the Chi square test). In the CLP group receiving anti-C5a antibodies, survival times

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were dramatically improved. By day 5, 50% of the animals survived, and no additional deaths were observed in the next 5 days. When the CLP group receiving preimmune IgG was compared to the group receiving anti-C5a IgG, the p values for 10 day survival were 0.012 and 0.022 by Chi Square Test and Fisher's exact test

5   respectively. Comparing the outcome of the CLP group receiving anti-C5a antibodies to the CLP group depleted of C3, p equaled 0.01 according to the Chi square test.

EXAMPLE 4

Anti-Rat C5a Antibodies Reduce Bacteremia In Septic Rats

This Example describes the *in vitro* detection and reduction of bacteremia in

10   septic rats. CLP-induced sepsis was generated in two different groups of rats according to the procedure of Example 1 above. One group (n=5) was treated intravenously with 400 µg of preimmune IgG, which was infused immediately after surgery. The second group (n=5) was treated intravenously with 400 µg of anti-C5a IgG (prepared according to Example 2), which was infused immediately after surgery.

15   A third group of sham operated rats (n=5) in which no cecal puncture was performed, were also included.

CLP-induced sepsis in rats is known to be associated with the development of bacteremia involving the presence of both aerobic and anaerobic bacteria in the blood [Deitch, E.A. *Schock* 9:1-11, (1997)]. Blood samples were obtained from these three

20   groups 36 hours after sham surgery or induction of CLP. This was done by drawing blood via the posterior vena cava after topical treatment of the puncture site with iodine swabsticks (Professional Disposables, Inc., Orangeburg, NY). Blood samples were placed in Isolator Microbial Tubes (Wampole, Inc.) and cultured on chocolate sheep blood agar plates (incubated aerobically in 5% CO_2) or on lysed blood agar

25   plates (incubated anaerobically). All plates were incubated for 96 hours prior to determination of colony counts (cfu). The presence of aerobic and anaerobic bacteria in blood was measured as cfu is shown in Figure 2.

In the sham operated group at 36 hours, cfu were not detectable. In the CLP group receiving preimmune IgG, the cfu value (x ± SEM) was 740 ± 328, while in the

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CLP group treated with anti-C5a antibodies, the cfu value was profoundly reduced, by 98% ($p<0.05$), to 18 ± 10 cfu. When subcultures of mixed aerobic or anaerobic bacteria obtained from the blood of CLP rats incubated in the presence of either preimmune IgG or anti-C5a IgG (each at 100 µg/ml), no reduction in cfu values were found, indicating that anti-C5a antibodies are not directly bacteriostatic.

Bacteremia was also assayed in the organs of both sham and CLP treated rats, which received either preimmune IgG or anti-C5a antibodies (prepared according to Example 2). Livers and spleens were obtained from these rats 36 hours after surgery. These tissues were homogenized and cfu/g of tissue was determined using procedures similar to those used for the blood samples described above. The data is shown in Figure 3, in which the cfu values for aerobic and anaerobic bacteria were arbitrarily aggregated. In all cases, there was a 1:1 ratio of these two classes of bacteria. In sham animals and in CLP animals treated with anti-C5a IgG, the cfu values were low in both liver and spleen. Between these two groups and in both organs, there were no statistically significant differences in cfu values. In marked contrast, CLP rats treated with preimmune IgG had very high cfu values ($3-11 \times 10^6$/g tissue).

EXAMPLE 5

Anti-Rat C5a Antibody Reduces C5a Binding to Neutrophils

This Example describes the binding of C5a peptide to neutrophils in the blood of septic rats, and ability of anti-rat C5a antibodies to reduce this binding. Blood neutrophils were obtained from both sham and CLP treated rats, which received either preimmune IgG or anti-C5a antibodies (prepared according to Example 2) at 12, 24 and 36 hours after CLP. Neutrophils were evaluated in flow cytometry for the surface content of C5a peptide using the procedure described below.

Whole blood was recovered from these rats, being drawn into syringes containing the anticoagulant ACD (Baxter Health Care Corp., Deerfield, IL.). Duplicate aliquots (250 µl) of cells were incubated with an equal volume of phosphate buffered saline. The phosphate buffer, pH 7.4, was made up with heat inactivated 1% fetal bovine serum and 0.1% NaN_3, containing 10 µg/ml of either anti-rat C5a

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polyclonal antibody (as used in Mulligan, M.S. *et al.*, *J. Clin. Invest.* 98:503-512, 1996) or irrelevant rabbit IgG control antibody (Jackson Laboratories, Ban Harbor, ME). The cells were incubated at 5°C for 30 minutes. Cells were washed once and red blood cells lysed with FACS solution (Becton Dickinson, San Jose, CA). Cells

5   were then washed and incubated with phycoerythrin labeled anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO). Cells were washed twice and suspended in 400 µl PBS containing 2% paraformaldehyde. Phycoerythrin intensity of gated populations (identified as forward versus side scatter light) was measured on a FACScan Flow Cytometry System (Becton Dickinson) in which 10,000 cells per gate were counted

10 and the amount of phycoerythrin analyzed using PC-LYSYS software (Becton Dickson).

The data collected from this procedure is shown in Table 4. Mean channel fluorescence (MCF) values for neutrophils obtained from sham operated rats or from CLP rats at 12 hours showed very low MCF values for C5a peptide. By 24 and 48

15 hours, neutrophils from CLP animals showed nearly 4 fold increases in binding of anti-C5a IgG, suggesting that these cells contained substantial amounts of C5a on their surfaces. Binding of preimmune IgG to neutrophils from CLP rats at 24 and 36 hours was very low (less than 10 MCF units). Thus, during CLP induced sepsis, blood neutrophils acquire C5a peptides on their surfaces. When C5a peptide content was

20 evaluated on blood neutrophils obtained from CLP rats pre-treated with 400 µg anti-C5a, there were consistent reductions in C5a content at 24 and 36 hours when compared to neutrophils from CLP rats pre-treated with preimmune IgG.

Table 4

Detection by Flow Cytometry of C5a Peptides on Blood

Neutrophils During Sepsis

Group	Mean Channel Fluorescence (mean ± SEM)			
	0 hour	12 hour	24 hour	36 hour
Sham	12.8 ± 1.48*			
CLP + preimmune IgG (400 µg)		11.9 ± 0.54	43.5 ± 1.42	45.6 ± 0.64
CLP + anti-C5a IgG (400 µg)		10.6 ± 0.37	32.6 ± 0.34	32.3 ± 2.74

* All displayed binding values represent results with rabbit anti-C5a IgG as detected with phycoerythrin labeled anti-rabbit IgG. The binding value of preimmune IgG was 4.45 ± 0.16. For each data point, n=4 and all samples were analyzed in quadruplicate.

EXAMPLE 6

Anti-Rat C5a Antibody Preserves H_2O_2 Production in Septic Rat Neutrophils

This Example describes the ability of anti-rat C5a antibody to preserve the H_2O_2 production of neutrophils from septic rats. Blood neutrophils were obtained from both sham and CLP treated rats, which received 400 µg of either preimmune IgG or anti-C5a antibodies (prepared according to Example 2) 36 hours after CLP or sham surgery. Neutrophil generation of H_2O_2 was assayed using the procedure described below.

Neutrophils were isolated from blood using dextran sedimentation and hypotonic red blood cell lysis. 7.5×10^5 cells were suspended in Hank's balanced salt solution (in a final volume of 1.0 ml) in the presence or absence of catalase (100 units/ml) in a final volume of 1.0 ml. Neutrophils were then stimulated for 1 hour at 37° with phorbol myristate acetate (PMA) at a concentration of 100 ng/ml.

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Stimulation was terminated by addition of 10% (vol/vol) trichloroacetic acid. After

removing precipitated protein by centrifugation (10 minutes at 500 x g), 10 mM

ferrous ammonium sulfate (0.2 ml) and 2.5 M potassium thiocyanate (0.1 ml) were

added to the sample. The presence of the ferrithiocyanate complex formed in the

5   presence of peroxide was measured at 480 nm and compared to a standard curve

generated using dilutions of stock H_2O_2.

The data collected from this procedure is shown in Figure 4. Very little H_2O_2

(circa 0.1 nmol) was produced in unstimulated blood neutrophils obtained from sham

rats or from CLP rats pre-treated with preimmune IgG or anti-C5a IgG. After PMA

10   stimulation, neutrophils from sham rats produced 3.1 ± 0.75 nmol H_2O_2. In CLP rats

treated with preimmune IgG, H_2O_2 production of PMA-stimulated neutrophils was

reduced by nearly 62%, to 1.25 ± 0.50 nmol. In striking contrast, blood neutrophils

from CLP animals treated with anti-C5a demonstrated full H_2O_2 generation, 3.58 ±

0.67 nmol after *in vitro* stimulation with PMA, indicating that treatment with anti-C5a

15   antibodies preserved this response in neutrophils from CLP rats.

EXAMPLE 7

C5a Peptides Reduce Chemotaxis of Human Neutrophils

This Example describes the ability of certain synthetic peptides representative

of regions of human C5a peptide to reduce the chemotactic response of human

20   neutrophils to human C5a peptide. Human neutrophils were isolated from human

blood by traditional Ficoll-Hypaque sedimentation techniques. Using standardized

methodology, neutrophils (5 x 10^6/ml) labeled with 1 μg/ml BCECF [2',7'-(2

carboxyethyl)-5-(and-6)-carboxyfluroscein, acetoxymethy ester) at 37°C for 30 min,

were applied to the upper compartments of chemotactic chambers and evaluated for

25   their motility responses to 10 nM human C5a peptide (SEQ ID NO:3) added to the

lower compartments. The two compartments were separated by a membrane with pore

sizes of 3 μm. Neutrophil chemotactic responses were quantitated by cytofluorometry.

Three different peptides, labelled A, M, and C, were also added separately to

the lower compartments, at a concentration of 1μM, in medium alone or together with

10 nM human recombinant C5a peptide. Peptide A (SEQ ID NO:4) represents residues 1-20 of human C5a peptide, peptide M (SEQ ID NO: 5) represents residues 21-40 of human C5a peptide, and peptide C (SEQ ID NO:6) represents residues 55-74 of human C5a peptide. As shown in Figure 5, the presence of 1μM from regions A,

5 M, or C, did not per se induce any chemotactic responses. However, the presence of 1μM of any of these three peptides with 10 μM human C5a resulted in significant reduction (approximately 20%) in the chemotactic responses of neutrophils. This data suggests that peptides from the N-terminal, mid-portion, and C-terminal regions of human C5a have the ability to compete functionally with intact C5a peptide, while

10 demonstrating no intrinsic chemotactic activity.

EXAMPLE 8

Neutrophil Chemotactic Activity of C5a Peptides Linked to KLH

This Example describes neutrophil chemotactic activity of peptides A, M, and C linked to keyholelimpet hemocyanin (KLH) employing the chemotactic assay described in Example 7. The peptide:KLH molar ratios employed were approximately

15 3:1. The chemotactic responses of neutrophils was evaluated to medium alone, to 10 nM recombinant C5a peptide (SEQ ID NO:3), and to the KLH-A, M, or C conjugates (SEQ ID NOS:4, 5, and 6 respectively), at the calculated synthetic peptide concentrations of 100 nM.

20 The data collected in this Example is shown in Figure 6. The A peptide KLH conjugate was the most chemotactically active compound when compared to C5a peptide, while the C peptide conjugate was almost as active. The M peptide conjugate revealed no chemotactic activity.

EXAMPLE 9

25 **Polyclonal Rabbit Anti-Human C5a Reactivity with Human C5a Peptide**

In this Example epitopes in human C5a peptide were evaluated for reactivity with commercially available rabbit polyclonal anti-human C5a antibodies (purchased from Calbiochem-Novabiochem Corp., San Diego, CA). Thirty-four (34) μg of this

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polyclonal anti-human C5a IgG was incubated with 20 μg KLH peptide conjugates (A, M, C, as described in Example 8) for 18 hours at 4°C. Treated and untreated antibodies were then evaluated for their ability to react with recombinant human C5a peptide by Western blot analysis. Fifty (50) ng C5a peptide was added to each lane, and electrophoresis was carried out. As shown in Figure 7, two banding patterns (one between the 6.5 and 14.3 kDa markers and the other near the 21.4 kDa marker) were found by Western blot analysis. When anti-C5a antibody was incubated with human recombinant C5a peptide, both bands disappeared in Western blots. Preabsorption of the antibody with C peptide abolished the slower band and nearly abolished the faster migrating band. Absorption with M peptide greatly diminished the intensity of both bands, while preabsorption with A peptide showed little evidence of removal of reactivity of the antibody with C5a peptide. This data suggests that the commercially available polyclonal rabbit antibody to human C5a peptide is most reactive with the C terminal region of C5a peptide (represented by peptide C), less reactive with the mid-region of C5a (represented by peptide M), and little, if at all, reactive with the N terminal region of human C5a (represented by peptide A).

EXAMPLE 10

Peptides Which Antagonize the Binding of Human C5a Peptide to Neutrophils

This Examples describes certain peptides which are able to antagonize the ability of human C5a peptide to bind to human neutrophils. Three different peptides, labelled A, M, and C, were used to antagonize the binding of ^{125}I-hC5a peptide to human neutrophils. Again, peptide A (SEQ ID NO:4) represents residues 1-20 of human C5a peptide, peptide M (SEQ ID NO: 5) represents residues 21-40 of human C5a peptide, and peptide C (SEQ ID NO:6) represents residues 55-74 of human C5a peptide.

Human peripheral blood neutrophils (1 x 10^7 cells/ml) were incubated in Hank's buffered saline plus 0.1% bovine serum albumin with both ^{125}I-labelled hC5a peptide (300μCi/nmol) and either peptide A, M, or C, in a final volume of 200 μl in a microfuge tube. The ratio of the shorter peptides (A, M, or C) to ^{125}I-labelled hC5a

peptide was 10:1. After incubation, cell suspension were layered over a 20% sucrose gradient and were sedimented by centrifugation at 11,000g. The tubes were then frozen on dry ice, followed by cutting the tips containing the pellet. Cell-bound label was then determined by placing the tips in a gamma counter.

5   As shown in Figure 9, peptides A and M were significantly ($p<0.05$) competitive in reducing the binding of hC5a peptide, whereas the C terminal peptide (peptide C) showed no statistically significant interference.

EXAMPLE 11

Screening Candidate Peptides For Useful Immunogens

10   This Example describes three methods which are employed in screening candidate C-terminal truncated C5a peptides for useful immunogens (*i.e.* which can be used to produce the anti-C5a antibodies of the present invention). One method involves screening C-terminal truncated C5a peptides which inhibit the chemotaxis of neutrophils. Another method involves screening peptides for the ability to antagonize

15   the binding of C5a peptides to neutrophils. A third method involves administering candidate C-terminal truncated C5a peptides to septic animals and monitoring their response.

        The first method, as described in Examples 7 and 8, is used to screen candidate C-terminal truncated C5a peptides which inhibit the chemotaxis of neutrophils. With

20   this method, human neutrophils are isolated from human blood by traditional Ficoll-Hypaque sedimentation techniques. Using standardized methodology, neutrophils are applied to the upper compartments of chemotactic chambers and evaluated for their motility responses to 10 nM human C5a peptide (SEQ ID NO:3) added to the lower compartments. The two compartments are separated by a membrane with pore sizes of

25   3 µm. Neutrophil chemotactic responses are quantitated by cytofluorometry.

        A candidate C-terminal truncated peptide (1µM), which may be linked to KLH, is added to the lower compartments in medium alone or together with 10 nM human recombinant C5a peptide. The chemotactic response of the neutrophils is then quantitated by cytofluorometry. One indication of a useful immunogen is if the

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candidate C-terminal truncated C5a peptide induces chemotaxis of the neutrophils as compared to human C5a peptide alone. Another indication of a useful immunogen is if the candidate C-terminal truncated C5a peptide inhibits chemotaxis of neutrophils when combined with human C5a peptide, as compared to human C5a peptide alone.

A second method, as described in Example 10, is used to screen candidate C-terminal truncated C5a peptides which antagonize the binding of ^{125}I-C5a peptide to neutrophils. In this Example, human neutrophils are incubated with human ^{125}I-C5a peptide and the candidate C-terminal truncated C5a peptide. Inhibition of ^{125}I-C5a peptide binding to the neutrophils by the candidate C-terminal truncated peptide indicates a potentially useful immunogen for the production of anti-C5a antibodies.

A third method employs the septic rats described in Example 1. In this Example, one group of CLP sepsis induced rats is administered 50 mg/kg of a candidate C-terminal C5a truncated peptide intravenously immediately after the CLP procedure, while a second group of CLP rats is used as a control. Survival rates and symptoms of sepsis are recorded over a ten day period. Candidate C-terminal truncated C5a peptides which reduce the symptoms of sepsis, and/or increase survival times as compared to the control group are considered potential immunogens for producing anti-C5a antibodies.

EXAMPLE 12

H_2O_2 Production of Human Neutrophils In The Presence of Short C5a Peptides

This Example describes the effect of human C5a peptide, and certain shorter synthetic human C5a peptides A, M, or C, to inhibit the H_2O_2 production of human neutrophils stimulated by phorbol myristate acetate (PMA). Again, peptide A (SEQ ID NO:4) represents residues 1-20 of human C5a peptide, peptide M (SEQ ID NO: 5) represents residues 21-40 of human C5a peptide, and peptide C (SEQ ID NO:6) represents residues 55-74 of human C5a peptide. Human neutrophils were isolated and then pretreated with C5a peptide or one of the shorter synthetic peptides (A, M, or C). Cells were then stimulated with PMA and the production of H_2O_2 was measured.

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As shown in Figure 10, C5a peptide and the shorter peptides alone have no effect on H_2O_2 production. However, PMA elicits a strong H_2O_2 response, which is inhibitable by the C5a peptide, and to varying degrees by peptides A, M, and C. Peptide M was shown to exert the strongest suppression of H_2O_2 production of all of the peptides (*See* Figure 10).

EXAMPLE 13

Preparation and Characterization of Anti-Human C5a Antibodies

This Example describes the production of anti-human C5a antibodies. A short human C5a peptide with the sequence CCYDGASVNNDETCEQRAAR (peptide M, SEQ ID NO:5) was obtained from Research Genetics (Huntsville Ala.) and coupled to keyhole limpet hemocyanin (KLH). The coupled peptide was used as an antigen to immunize rabbits. After several injections, the antibody was recovered by affinity purification.

A Western Blot was then used to demonstrate that these anti-human C5a antibodies are specific for human C5a peptide, and not rat C5a peptide. Briefly, recombinant human and rat C5a peptide were run in parallel on a gel, and transferred to a filter. The filter was blocked, and then probed with the anti-human C5a antibody. The resulting signal revealed that the anti-human C5a antibody was only able to recongnize human C5a peptide, and not rat C5a peptide (*See* Figure 11).

An additional Western blot was also performed in order to determine if polyclonal anit-human antibodies (commercially available from Calbiochem) shared this type of specificity. Again, recombinant human and rat C5a peptide were run in parallel on a gel, and transferred to a filter. The filter was blocked, and then probed with the commercially available polyclonal anti-C5a antibody. The resulting signal revealed that the polyclonal anti-human C5a antibody was able to recognize both human and rat C5a peptide (*See* Figure 11). As such, it is clear that the commercially available polyclonal anti-human C5a antibody does not have the same type of specificity as the anti-human C5a antibody discussed above.

EXAMPLE 14

Therapeutic Use of Anti-Human C5a Antibodies to Treat Sepsis In Humans

Anti-C5a antibodies may be used prophylactically or therapeutically to treat sepsis in humans. Individuals at risk of contracting sepsis, particularly patients undergoing surgery, or those with sepsis may be administered an effective amount of anti-C5a antibodies to prevent or reduce the severity of the disease. A typical treatment regimen would consist of administering 5-10 mg of antibody per kilogram of patient body weight. Prophylactically the dose would be given just prior to surgery, and repeated at least once immediately thereafter. Therapeutically the dose would be given every 24-48 hours until remission of the disease is apparent. The initial therapeutic dose would be 25 mg per kilogram of patient body weight, and then reduced to 5-10 mg per kilogram. The antibody may be administered by any number of routes, but the preferred route of administration is intravenously.

EXAMPLE 15

Sepsis Rescue Following Delayed Administration of Anti-Rat C5a

Rats (see, Fig. 12A) subjected to CLP were treated intravenously at hour 0 (e.g. at the time of CLP) with 600 ug rabbit IgG containing antibody to SEQ ID NO:2 or SEQ ID NO: 75 of rat C5a. Treatment with either of these anti-C5a preparations facilitated survival rates at day 10, after CLP, between 85-90% (in each group, n = 10 to 12). In contrast, CLP rats treated with preimmine IgG (pre IgG) only showed survival rates of approximately 30%. The difference in post CLP survival, between the animals treated (at post CLP hour 0), with antibody to SEQ ID NO:2 or SEQ ID NO:75, as compared to animals only treated with "pre-IgG", is statistically significant ($p < 0.05$).

Rats were also treated intravenously with anti-C5a 6 hours after CLP (see, Fig. 12B). Even 6 hours after cecal puncture, administration of anti-C5a preparations to SEQ ID NO:2 or SEQ ID NO:75 regions of rat C5a facilitated therapeutic improvement. These survival rates (approximately 60-70%) of animals treated with

anti-C5a preparations to SEQ ID NO:2 or SEQ ID NO:75 regions of rat C5a are approximately three time that of the "pre IgG" control group at day 10. That is to say, rats treated with "pre IgG" presented survival rate of approximately 20% at day 10. Once again, the difference in post CLP survival, between the animals treated (at post CLP hour 6), with antibody to SEQ ID NO:2 and SEQ ID NO:75, as compared to animals only treated with "pre-IgG", is statistically significant ($p < 0.05$).

Rats were also treated intravenously with anti-C5a 12 hours after CLP (see, Fig. 12C). Even 12 hours after cecal puncture, administration of anti-C5a preparations to SEQ ID NO:2 or SEQ ID NO:75 regions of rat C5a still facilitated significant therapeutic improvement. These survival rates (approximately 40-50%) of animals treated with anti-C5a preparations to SEQ ID NO:2 or SEQ ID NO:75 regions of rat C5a are approximately three times that of the "pre IgG" control group at day 10. That is to say, rats treated with "pre IgG" presented survival rate of less that 20% at day 10. Once again, the difference in post CLP survival, between the animals treated (at post CLP hour 12), with antibody to SEQ ID NO:2 and SEQ ID NO:75, as compared to animals only treated with "pre-IgG", is statistically significant ($p < 0.05$).

These data demonstrate that, when infusion of anti-C5a antibodies into rats with CLP-induced sepsis is delayed 6-12 hours, rats may be rescued from their septic condition. That is to say, 6-12 hours after cecal puncture rats are already in an advanced "hyperdynamic" state (elevated heart rate and breathing, reduced blood CO_2, etc.) and are beginning to show evidence of multi-organ failure (elevated transaminases, creatinine, and blood urea nitrogen, indicative of early failure in renal and hepatic function). These physiological events in the rat model of sepsis appear to be very early in onset, much faster than those appearing in humans in which typically similar events of sepsis take place over a 3 - 4 day period. These data suggest that a human presenting any symptom of sepsis has a "therapeutic window" of several days during which the administration of anti-C5a antibodies will cause an abatement of septic symptoms.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described

method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific

5   embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in bio-chemistry, immunology, chemistry, molecular biology, the medical profession or related fields are intended to be within the scope of the following claims.